

## Claims

- [c1] 1. A method for real-time detecting and quantifying a nucleic acid template in a PCR mixture comprising the steps of
- thermally cycling the PCR mixture, wherein the PCR mixture comprises a thermostable polymerase, the nucleic acid template, primers to amplify at least one amplicon from the nucleic acid template, and a double stranded DNA dye, wherein the amplicon has a melting temperature of  $T_m$ ;
  - obtaining cycle by cycle a pre- $T_m$  emission at a MT below the  $T_m$  and a post- $T_m$  emission at the a MT above the  $T_m$ ;
  - determining cycle by cycle an emission amount of the amplicon, which is the difference between the pre- $T_m$  emission and the post- $T_m$  emission.
- [c2] 2. The method of claim 1 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.
- [c3] 3. The method of claim 2 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.
- [c4] 4. The method of claim 1 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.
- [c5] 5. The method of claims 4 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c6] 6. The method of claim 1 wherein the MT below the  $T_m$  is 0.25 °C below, 0.5 °C below, 1.0 °C below, 1.5 °C below, or 2.0 °C below the  $T_m$ .

[c7] 7. The method of claim 1 wherein the MT above the  $T_m$  is 0.25 °C above, 0.5 °C above, 1.0 °C above, 1.5 °C above, or 2.0 °C above the  $T_m$ .

[c8] 8. The method of claim 1 wherein the emission amount of the amplicon is obtained through a computer program which performs a calculation of subtracting the pre- $T_m$  emission from the post- $T_m$  emission or the post- $T_m$  emission from the pre- $T_m$  emission.

[c9] 9. A method for real-time detecting and quantifying a first nucleic acid template and a second nucleic acid template in a PCR mixture comprising the steps of

a) thermally cycling a PCR mixture wherein the PCR mixture comprises a thermostable polymerase, a double stranded DNA dye, the first template and the second template, primers for amplifying a first amplicon from the first template and a second amplicon from the second template, and wherein the first amplicon has a first  $T_m$  and the second amplicon has a second  $T_m$  and the first  $T_m$  is less than the second  $T_m$ ;

b) obtaining cycle by cycle a first emission at a first MT between an annealing/extension temperature and the first  $T_m$  and a second emission at a second MT between the first  $T_m$  and the second  $T_m$ ;

c) determining cycle by cycle a first emission amount of the first amplicon which is the difference between the first emission and the second

emission, and a second emission amount of the second amplicon which is the second emission.

[c10] 10. The method of claim 9 further comprising a step of obtaining cycle by cycle a third emission at a third MT between the second  $T_m$  and a total denaturing temperature, wherein the second emission amount is the difference between the second emission and the third emission.

[c11] 11. The method of claim 9 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c12] 12. The method of claim 11 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c13] 13. The method of claim 9 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.

[c14] 14. The method of claims 13 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c15] 15. The method of claim 9 wherein the first MT is  $0.25^{\circ}\text{C}$  below the first  $T_m$ ,  $0.5^{\circ}\text{C}$  below the first  $T_m$ ,  $1.0^{\circ}\text{C}$  below the first  $T_m$ ,  $1.5^{\circ}\text{C}$  below the first  $T_m$ , or  $2.0^{\circ}\text{C}$  below the first  $T_m$ , and wherein the first MT is higher than the annealing temperature.

[c16] 16. The method of claim 9 wherein the second MT is  $0.25^{\circ}\text{C}$  below the second  $T_m$ ,  $0.5^{\circ}\text{C}$  below the second  $T_m$ ,  $1.0^{\circ}\text{C}$  below the second  $T_m$ ,  $1.5^{\circ}\text{C}$

below the second  $T_m$ , or  $2.0^{\circ}\text{C}$  below the second  $T_m$ , and wherein the second MT is higher than the first  $T_m$ .

[c17] 17. The method of claim 9 wherein the second MT is  $0.25^{\circ}\text{C}$  above the first  $T_m$ ,  $0.5^{\circ}\text{C}$  above the first  $T_m$ ,  $1.0^{\circ}\text{C}$  above the first  $T_m$ ,  $1.5^{\circ}\text{C}$  above the first  $T_m$ , or  $2.0^{\circ}\text{C}$  above the first  $T_m$ , and wherein the second MT is less than the second  $T_m$ .

[c18] 18. The method of claim 9 wherein the second MT is the first  $T_m + 0.25^{\circ}\text{C} < \text{the second MT} < \text{the second } T_m - 0.25^{\circ}\text{C}$ , the first  $T_m + 0.5^{\circ}\text{C} < \text{the second MT} < \text{the second } T_m - 0.5^{\circ}\text{C}$ , the first  $T_m + 1.0^{\circ}\text{C} < \text{the second MT} < \text{the second } T_m - 1.0^{\circ}\text{C}$ , the first  $T_m + 1.5^{\circ}\text{C} < \text{the second MT} < \text{the second } T_m - 1.5^{\circ}\text{C}$ , or the first  $T_m + 2.0^{\circ}\text{C} < \text{the second MT} < \text{the second } T_m - 2.0^{\circ}\text{C}$ .

[c19] 19. The method of claim 10 wherein the third MT is  $0.25^{\circ}\text{C}$  above the second  $T_m$ ,  $0.5^{\circ}\text{C}$  above the second  $T_m$ ,  $1.0^{\circ}\text{C}$  above the second  $T_m$ ,  $1.5^{\circ}\text{C}$  above the second  $T_m$ , or  $2.0^{\circ}\text{C}$  above the second  $T_m$ , and wherein the third MT is less than the total denaturing temperature.

[c20] 20. The method of claim 9 wherein the emission amount of the first amplicon is obtained through a computer program performing a calculation of subtracting the first emission from the second emission or subtracting the second emission from the first emission.

[c21] 21. A method for real-time detecting and quantifying a first nucleic acid template and a second nucleic acid template in a PCR mixture comprising the steps of:

- a) thermally cycling a PCR mixture wherein the PCR mixture comprises a thermostable polymerase, a double stranded DNA dye, the first template and the second template, primers for amplifying a first

amplicon from the first template and a second amplicon from the second template, and wherein the first amplicon has a first  $T_m$  and the second amplicon has a second  $T_m$  and the first  $T_m$  is less than the second  $T_m$ ;

- b) obtaining cycle by cycle a first pre- $T_m$  emission at a MT below the first  $T_m$  and a first post- $T_m$  emission at the a MT above the first  $T_m$  and a second pre- $T_m$  emission at a MT below the second  $T_m$  and a second post- $T_m$  emission at the a MT above the second  $T_m$ ;
- c) determining cycle by cycle a first emission amount of the first amplicon which is the difference between the first pre- $T_m$  emission and the first post- $T_m$  emission; and a second emission amount of the second amplicon which is the difference between the second pre- $T_m$  emission and the second post- $T_m$  emission.

[c22] 22. The method of claim 21 wherein the double stranded DNA dye is a double stranded DNA intercalating dye

[c23] 23. The method of claim 22 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c24] 24. The method of claim 21 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.

[c25] 25. The method of claims 24 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c26] 26. The method of claim 21 wherein the MT below the first  $T_m$  and/or the second  $T_m$  are 0.25 °C below, 0.5 °C below, 1.0 °C below, 1.5 °C below, or 2.0 °C below.

[c27] 27. The method of claim 21 wherein the MT above the first  $T_m$  and/or the second  $T_m$  are 0.25 °C above, 0.5 °C above, 1.0 °C above, 1.5 °C above, or 2.0 °C above.

[c28] 28. The method of claim 21 wherein the emission amount of the amplicons is obtained through a computer program performing the calculation of subtracting the pre- $T_m$  emission from the post- $T_m$  emission or subtracting the post- $T_m$  emission from the pre- $T_m$  emission.

[c29] 29. A method for real-time detecting and quantifying a total of  $n$  nucleic acid templates in a PCR mixture comprising the steps of:

a) thermally cycling a PCR mixture, wherein the PCR mixture comprises a thermostable polymerase, nucleic acid templates including  $n$  nucleic acid templates, primers for amplifying  $n$  amplicons, and a double stranded DNA dye;

b) obtaining cycle by cycle a MT<sub>k</sub> emission at MT<sub>k</sub> and MT<sub>(k+1)</sub>, wherein  $T_{m(k-1)} < MT_k < T_{mk} < MT_{(k+1)} < T_{m(k+1)}$ , T<sub>mk</sub> is the  $T_m$  of a  $k$ th amplicon, T<sub>m(k-1)</sub> is the  $T_m$  of a  $(k-1)$ th amplicon except that T<sub>m(k-1)</sub> is an annealing and/or an extension temperature when  $k=1$ , T<sub>m(k+1)</sub> is the  $T_m$  of a  $(k+1)$ th amplicon except that T<sub>m(n+1)</sub> is a total denaturing temperature when  $k=n$ , and  $k$  and  $n$  are positive integers,  $1 \leq k \leq n$ , and  $n \geq 2$ ;

c) determining cycle by cycle an emission amount of the  $k$ th amplicon which is the difference between the  $MT_k$  emission and the  $MT_{(k+1)}$  emission.

[c30] 30. The method of claim 29 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c31] 31. The method of claim 30 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c32] 32. The method of claim 29 wherein the double stranded DNA dye is a primer-based double stranded DNA dye that is covalently linked to the primers.

[c33] 33. The method of claims 32 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c34] 34. The method of claim 29 wherein  $T_{m(k-1)} + 0.25^{\circ}\text{C} < MT_k < T_{mk}$ ,  $T_{m(k-1)} + 0.5^{\circ}\text{C} < MT_k < T_{mk}$ ,  $T_{m(k-1)} + 1.0^{\circ}\text{C} < MT_k < T_{mk}$ ,  $T_{m(k-1)} + 1.5^{\circ}\text{C} < MT_k < T_{mk}$ , or  $T_{m(k-1)} + 2.0^{\circ}\text{C} < MT_k < T_{mk}$ .

[c35] 35. The method of claim 29 wherein  $T_{mk} + 0.25^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 0.5^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 1.0^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 1.5^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 2.0^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)}$ .

[c36] 36. The method of claim 29 wherein  $T_{m(k-1)} < MT_k < T_{mk} - 0.25^{\circ}\text{C}$ ,  $T_{m(k-1)} < MT_k < T_{mk} - 0.5^{\circ}\text{C}$ ,  $T_{m(k-1)} < MT_k < T_{mk} - 1.0^{\circ}\text{C}$ ,  $T_{m(k-1)} < MT_k < T_{mk} - 1.5^{\circ}\text{C}$ , or  $T_{m(k-1)} < MT_k < T_{mk} - 2.0^{\circ}\text{C}$ .

- [c37]       37. The method of claim 29 wherein  $T_{mk} < MT_{(k+1)} < T_{m(k+1)} - 0.25^{\circ}\text{C}$ ,  $T_{mk} < MT_{(k+1)} < T_{m(k+1)} - 0.5^{\circ}\text{C}$ ,  $T_{mk} < MT_{(k+1)} < T_{m(k+1)} - 1.0^{\circ}\text{C}$ ,  $T_{mk} < MT_{(k+1)} < T_{m(k+1)} - 1.5^{\circ}\text{C}$ ,  $T_{mk} < MT_{(k+1)} < T_{m(k+1)} - 2.0^{\circ}\text{C}$ .
- [c38]       38. The method of claim 29 wherein  $T_{m(k-1)} + 0.25^{\circ}\text{C} < MT_k < T_{mk} - 0.25^{\circ}\text{C}$ ,  $T_{m(k-1)} + 0.5^{\circ}\text{C} < MT_k < T_{mk} - 0.5^{\circ}\text{C}$ ,  $T_{m(k-1)} + 1.0^{\circ}\text{C} < MT_k < T_{mk} - 1.0^{\circ}\text{C}$ ,  $T_{m(k-1)} + 1.5^{\circ}\text{C} < MT_k < T_{mk} - 1.5^{\circ}\text{C}$  or  $T_{m(k-1)} + 2.0^{\circ}\text{C} < MT_k < T_{mk} - 2.0^{\circ}\text{C}$ .
- [c39]       39. The method of claim 29 wherein  $T_{mk} + 0.25^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)} - 0.25^{\circ}\text{C}$ ,  $T_{mk} + 0.5^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)} - 0.5^{\circ}\text{C}$ ,  $T_{mk} + 1.0^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)} - 1.0^{\circ}\text{C}$ ,  $T_{mk} + 1.5^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)} - 1.5^{\circ}\text{C}$ , or  $T_{mk} + 2.0^{\circ}\text{C} < MT_{(k+1)} < T_{m(k+1)} - 2.0^{\circ}\text{C}$ .
- [c40]       40. The method of claim 29 wherein  $2 \leq n \leq 35$ ,  $2 \leq n \leq 18$ ,  $2 \leq n \leq 10$ ,  $2 \leq n \leq 7$ , or  $2 \leq n \leq 5$ .
- [c41]       41. The method of claim 40 wherein  $n = 2, 3, 4$ , or  $5$ .
- [c42]       42. The method of claim 29 wherein the PCR mixture further comprises a FRET based probe.
- [c43]       43. The method of claim 42 wherein the FRET based probe is selected from the group consisting of a Taqman probe, a double-dye oligonucleotide probe, an Eclipse probe, a Molecular Beacon probe, a Scorpion probe, a Hybridization probe, a ResonSense probe, a Light-up probe, and a Hy-Beacon probe.
- [c44]       44. The method of claim 29 wherein the PCR mixture further comprises a second primer-based double stranded DNA dye that emits differently from the double stranded DNA dye.

[c45] 45. The method of claim 29 wherein the emission amount of the  $k$ th amplicon is obtained through a computer program performing the subtraction of  $MT_k$  emission from  $MT_{(k+1)}$  emission or the subtraction of the  $MT_{(k+1)}$  emission from  $MT_k$  emission.

[c46] 46. A method for detecting and quantifying a total of  $n$  nucleic acid templates in multiplex real-time PCR comprising the steps of:

a) thermally cycling a PCR mixture, wherein the PCR mixture comprises a thermostable polymerase, nucleic acid templates including  $n$  nucleic acid templates, primers for amplifying  $n$  amplicons, and a double stranded DNA dye;

b) obtaining cycle by cycle a pre- $T_{mk}$  emission of the  $k$ th amplicon at a MT between  $T_{m(k-1)}$  and  $T_{mk}$  and a post- $T_{mk}$  emission of the  $k$ th amplicon at a MT between  $T_{mk}$  and  $T_{m(k+1)}$ , wherein  $T_{m(k-1)} < T_{mk} < T_{m(k+1)}$ ,  $T_{mk}$  is the  $T_m$  of a  $k$ th amplicon,  $T_{m(k-1)}$  is the  $T_m$  of a  $(k-1)$ th amplicon except that  $T_{m(k-1)}$  is an annealing and/or an extension temperature when  $k=1$ ,  $T_{m(k+1)}$  is the  $T_m$  of a  $(k+1)$ th amplicon except that  $T_{m(n+1)}$  is a total denaturing temperature when  $k=n$ , and  $k$  and  $n$  are positive integers,  $1 \leq k \leq n$ , and  $n \geq 2$ ;

c) determining cycle by cycle an emission amount of the  $k$ th amplicon which is the difference between the pre- $T_{mk}$  emission and the post- $T_{mk}$  emission.

[c47] 47. The method of claim 46 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

- [c48] 48. The method of claim 47 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.
- [c49] 49. The method of claim 46 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.
- [c50] 50. The method of claims 49 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.
- [c51] 51. The method of claim 46 wherein the MT between  $T_{m(k-1)}$  and  $T_{mk}$  is  $T_{m(k-1)} + 0.25^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk}$ ,  $T_{m(k-1)} + 0.5^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk}$ ,  $T_{m(k-1)} + 1.0^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk}$ ,  $T_{m(k-1)} + 1.5^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk}$ , or  $T_{m(k-1)} + 2.0^{\circ}\text{C} < MT_k < T_{mk}$ .
- [c52] 52. The method of claim 46 wherein the MT between  $T_{mk}$  and  $T_{m(k+1)}$  is  $T_{mk} + 0.25^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 0.5^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 1.0^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 1.5^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)}$ ,  $T_{mk} + 2.0^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)}$ .
- [c53] 53. The method of claim 46 wherein the MT between  $T_{m(k-1)}$  and  $T_{mk}$  is  $T_{m(k-1)} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 0.25^{\circ}\text{C}$ ,  $T_{m(k-1)} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 0.5^{\circ}\text{C}$ ,  $T_{m(k-1)} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 1.0^{\circ}\text{C}$ ,  $T_{m(k-1)} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 1.5^{\circ}\text{C}$ , or  $T_{m(k-1)} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 2.0^{\circ}\text{C}$ .

- [c54] 54. The method of claim 46 wherein the MT between  $T_{mk}$  and  $T_{m(k+1)}$  is  $T_{mk} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 0.25^{\circ}\text{C}$ ,  $T_{mk} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 0.5^{\circ}\text{C}$ ,  $T_{mk} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 1.0^{\circ}\text{C}$ ,  $T_{mk} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 1.5^{\circ}\text{C}$ ,  $T_{mk} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 2.0^{\circ}\text{C}$ .
- [c55] 55.. The method of claim 46 wherein the MT between  $T_{m(k-1)}$  and  $T_{mk}$  is  $T_{m(k-1)} + 0.25^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 0.25^{\circ}\text{C}$ ,  $T_{m(k-1)} + 0.5^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 0.5^{\circ}\text{C}$ ,  $T_{m(k-1)} + 1.0^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 1.0^{\circ}\text{C}$ ,  $T_{m(k-1)} + 1.5^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 1.5^{\circ}\text{C}$  or  $T_{m(k-1)} + 2.0^{\circ}\text{C} <$  the MT between  $T_{m(k-1)}$  and  $T_{mk} < T_{mk} - 2.0^{\circ}\text{C}$ .
- [c56] 56. The method of claim 46 wherein the MT between  $T_{mk}$  and  $T_{m(k+1)}$  is  $T_{mk} + 0.25^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 0.25^{\circ}\text{C}$ ,  $T_{mk} + 0.5^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 0.5^{\circ}\text{C}$ ,  $T_{mk} + 1.0^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 1.0^{\circ}\text{C}$ ,  $T_{mk} + 1.5^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 1.5^{\circ}\text{C}$ , or  $T_{mk} + 2.0^{\circ}\text{C} <$  the MT between  $T_{mk}$  and  $T_{m(k+1)} < T_{m(k+1)} - 2.0^{\circ}\text{C}$ .
- [c57] 57. The method of claim 46 wherein  $2 \leq n \leq 35$ ,  $2 \leq n \leq 18$ ,  $2 \leq n \leq 10$ ,  $2 \leq n \leq 7$ , or  $2 \leq n \leq 5$ .
- [c58] 58 The method of claim 46 wherein the PCR mixture further comprises a FRET based probe.
- [c59] 59. The method of claim 46 wherein the FRET based probe is selected from the group consisting of a Taqman probe, a double-dye oligonucleotide probe, an Eclipse probe, a Molecular Beacon probe, a Scorpion probe, a Hybridization probe, a ResonSense probe, a Light-up probe, and a Hy-Beacon probe.

- [c60] 60. The method of claim 46 wherein the PCR mixture further comprises a second primer-based double stranded DNA dye that emits differently from the double stranded DNA dye.
- [c61] 61. The method of claim 46 wherein the emission amount of the  $k$ th amplicon is obtained through a computer program performing the subtraction of the pre- $T_{mk}$  emission from the post- $T_{mk}$  emission or the subtraction of the post- $T_{mk}$  emission from the pre- $T_{mk}$  emission
- [c62] 62. A computer software program for quantifying a real-time PCR amplicon which, when executed by a computer processor, performs the subtraction of a pre- $T_m$  emission from a post- $T_m$  emission or the subtraction of the post- $T_m$  emission from the pre- $T_m$  emission.
- [c63] 63. The computer software program of claim 62 wherein the emission was obtained from a double stranded DNA dye.
- [c64] 64. The computer software program of claim 62 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.
- [c65] 65. The computer software program of claim 64 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.
- [c66] 66. The computer software program of claim 62 wherein the double stranded DNA dye is a primer-based double stranded DNA dye that is covalently linked to the primers.
- [c67] 67. The computer software program of claim 66 wherein the primer-based double stranded DNA dye is selected from the group consisting of [41743-8001/LA032110.024.024]

fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

- [c68] 68. The computer software program of claim 62 wherein a pre- $T_m$  emission is obtained at a MT below the  $T_m$  of the amplicon and a post- $T_m$  emission is obtained at a MT above the  $T_m$ .
- [c69] 69. The computer software program of claim 68 wherein the MT below the  $T_m$  is 0.25 °C below, 0.5 °C below, 1.0 °C below, 1.5 °C below, or 2.0 °C below the  $T_m$ .
- [c70] 70. The computer software program of claim 68 wherein the MT above the  $T_m$  is 0.25 °C above, 0.5 °C above, 1.0 °C above, 1.5 °C above, or 2.0 °C above the  $T_m$ .
- [c71] 71. The computer software program of claim 62 which is stored and/or executed in a PCR instrument.
- [c72] 72. The computer software program of claim 62 which is stored and/or executed in a computer connected to a PCR instrument.
- [c73] 73. A computer program product comprising a computer memory having a computer software program, wherein the computer software program, when executed by a computer processor, performs the subtraction of a pre- $T_m$  emission from a post- $T_m$  emission or the subtraction of the post- $T_m$  emission from the pre- $T_m$  emission.
- [c74] 74. The computer program product of claim 73 wherein the emission was obtained from a double stranded DNA dye.

- [c75] 75. The computer program product of claim 73 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.
- [c76] 76. The computer program product of claim 75 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.
- [c77] 77. The computer program product of claim 73 wherein the double stranded DNA dye is a primer-based double stranded DNA dye that is covalently linked to the primers.
- [c78] 78. The computer program product of claim 77 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.
- [c79] 79. The computer program product of claim 73 wherein a pre- $T_m$  emission is obtained at a MT below the  $T_m$  of the amplicon and a post- $T_m$  emission is obtained at a MT above the  $T_m$ .
- [c80] 80. The computer program product of claim 79 wherein the MT below the  $T_m$  is 0.25 °C below, 0.5 °C below, 1.0 °C below, 1.5 °C below, or 2.0 °C below the  $T_m$ .
- [c81] 81. The computer program product of claim 79 wherein the MT above the  $T_m$  is 0.25 °C above, 0.5 °C above, 1.0 °C above, 1.5 °C above, or 2.0 °C above the  $T_m$ .
- [c82] 82. The computer program product of claim 73 which is stored and/or executed in a PCR instrument.

- [c83]        83. The computer program product of claim 73 which is stored and/or executed in a computer connected to a PCR instrument.
- [c84]        84. A PCR instrument comprising the computer program product of claim 73.